

Exposure to α -Copaene and α -Copaene-Containing Oils Enhances Mating Success of Male Mediterranean Fruit Flies (Diptera: Tephritidae)

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ABSTRACT Previous research revealed that exposure to the synthetic attractant trimedlure increased the mating success of male Mediterranean fruit flies, *Ceratitis capitata* (Wiedemann). In the current study, I investigated whether a similar effect resulted from male exposure to α -copaene, a naturally occurring compound found in many host plants, and two α -copaene-containing essential oils, angelica seed oil (*Angelica archangelica* L.) and ginger root oil, (*Zingiber officinale* Roscoe). For all three substances, mature males were exposed to 20 μ l of the attractant over a 6-h period and then held 2 d before testing. In field-cage trials, treated males (exposed to attractants) obtained significantly more matings than control males (no exposure) for all three substances. In an additional experiment with ginger root oil, treated males prevented from contacting the oil directly (i.e., exposed to the odor only) still exhibited a mating advantage over control males. Discussion centers on the influence of α -copaene-bearing plants on the mating system of *C. capitata* and the possibility of using attractants in prerelease exposure of males to increase the effectiveness of sterile insect release programs.

KEY WORDS *Ceratitis capitata*, attractant, α -copaene, mating behavior

VOLATILES FROM HOST plants interact with the pheromone communication of phytophagous insects in three main ways. First, they may enhance the attraction of the receiving sex to olfactory signals. Studies on a variety of taxa, including beetles (Bartelt et al. 1992, Giblin-Davis et al. 1997), moths (Hendrikse and Vos-Bunnemeyer 1987, Landolt et al. 1994), and flies (Dickens et al. 1990, Landolt et al. 1992) have demonstrated increased attraction to aggregation or sex pheromones in the presence of host-plant odor or green-leaf volatiles (but see Guldemon et al. 1993 for an exception). Second, host plant odors may be used in pheromone synthesis. In a well-known case, males of certain bark beetles convert vapors of the host monoterpene myrcene into components of the sex pheromone (Hughes 1974, Byers 1982). Third, host plant odors may stimulate pheromone production (Raina et al. 1992) or release (Hendrikse and Vos-Bunnemeyer 1987, McNeil and Delisle 1989). These effects may act concurrently, and isolating them may be problematic. For example, Landolt et al. (1994) observed greater responsiveness of male moths to female pheromone in the presence of host plant odors. However, additional experiments revealed that this result reflected increased signaling by females in the presence of the host plant odor rather than synergism between plant and female odors.

Pheromones are an important component of the mating systems of many species of tephritid fruit flies (Jang and Light 1996). However, the interface be-

tween host-plant chemistry and olfactory-sexual communication in the Tephritidae has received little attention, and existing studies (Dickens et al. 1990, Landolt et al. 1992) have dealt solely with the influence of plant odors on female responsiveness to male pheromones. The fact that males of many tephritid species are strongly attracted to the odors of particular natural or synthetic compounds (Jang and Light 1996) suggests a potential link between plant chemistry and male pheromone release or production. This interaction has been best studied in the genus *Bactrocera* by Nishida and his colleagues (Nishida et al. 1988, 1993, 1997; Tan and Nishida 2000), who identified metabolites of natural attractants (from nonhost plants) in the pheromone glands of male *B. dorsalis* (Hendel) and *B. cucurbitae* (Coquillett) following ingestion of the natural attractants. Behavioral studies (Shelly and Dewire 1994; Shelly and Villalobos 1995; Shelly et al. 1996a; Tan and Nishida 1996, 1998; Shelly 2000a) on these and closely related species further revealed that males that fed on attractants displayed greater signaling activity and had higher mating success than control males.

Chemical communication plays an important role in the lek behavior of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann). Males defend individual leaves as mating territories and, while perching, emit a pheromone attractive to females by everting their rectal epithelium and expanding their abdominal pleura (a behavior hereafter termed 'pheromone-calling' or, simply, 'calling'; Arita and Kaneshiro 1986).

Previous studies (Shelly et al. 1996b, Shelly 1999) have shown that males exposed to trimedlure, a synthetic male lure used routinely in control programs (Dowell and Penrose 1995), increased the level of pheromone calling, which presumably contributed to the enhanced mating success observed for trimedlure-exposed males. This effect was short-lived, however, and was most pronounced within 24 h of exposure (Shelly et al. 1996b). Unlike *Bactrocera*, males of the Mediterranean fruit fly do not feed on trimedlure, and the odor alone stimulated increased pheromone-calling.

Here I have investigated whether exposure to certain natural plant products, in particular the sesquiterpene hydrocarbon α -copaene and two essential oils (angelica seed oil, *Angelica archangelica* L., and ginger root oil, *Zingiber officinale* Roscoe) that contain α -copaene, similarly enhance mating success of male *C. capitata*. In an additional experiment, I examined whether exposure to ginger root oil affected the incidence of male calling and correspondingly female visitation to male calling sites. Many studies (Steiner et al. 1957; McInnis and Warthen 1988; Flath 1994a, 1994b; Nishida et al. 2000) have demonstrated the attraction of male *C. capitata* to α -copaene or α -copaene-containing substances. Additionally, α -copaene has been found in a wide variety of plants, including several important hosts of *C. capitata* such as orange, *Citrus sinensis* (L.); lemon, *C. limon* (L.); guava, *Psidium guajava* L.; papaya, *Carica papaya* L.; and mango, *Mangifera indica* L. (Nishida et al. 2000 and references therein). Based on these findings, it has been suggested that male attraction to α -copaene evolved primarily as a rendezvous mechanism to facilitate location of host plants and the subsequent formation of male aggregations or leks (Metcalf 1979, Nishida et al. 2000). As described below, the current study supports the alternative (but not mutually exclusive) explanation that males are attracted to α -copaene, because they derive a mating benefit from exposure to this chemical.

Materials and Methods

Study Animals. The flies used in the α -copaene and angelica seed oil mating trials derived from a laboratory colony started with 400–500 adults reared from loquat, *Eriobotrya japonica* Thunb., collected on Maui. The flies used in the ginger root oil experiments derived from a laboratory colony started with 200–300 adults reared from Jerusalem cherry, *Solanum pseudocapsicum* L., collected in Hawaii Volcanoes National Park. Adults were held in screen cages and provided with a sugar-protein mixture (3:1 by weight), water, and oviposition substrate (perforated plastic vials containing small sponges soaked in lemon juice). Eggs were placed on standard larval medium (Tanaka et al. 1969) in plastic containers placed over vermiculite for pupation. Adults used in the experiments were separated by sex within 24 h of eclosion, well before reaching sexual maturity at 5–7 d of age, and kept in 5-liter buckets (100–125 flies per bucket) with ample food and water. The flies were maintained at 20–24°C and

65–85% RH and received both natural and artificial light with a photoperiod of 12:12 (L:D) h. The generation time under these conditions was approximately 4 wk. Flies used in the α -copaene and angelica seed oil mating trials were 15 generations removed from the wild, while the colony used in the ginger root oil experiments was maintained in the laboratory for 12 generations before study.

Male Attractants. The α -copaene used in the current study (92% purity; enantiomeric purity undetermined) was prepared from angelica seed oil following the procedures outlined by Nishida et al. (2000) and diluted with hexane at a concentration of 5 μ g to 1 μ l hexane. Angelica seed oil and ginger root oil were obtained commercially from R.W.B. Starke & Sons, Eye, Suffolk, UK, and from Citrus and Allied Essences, Lake Success, NY, respectively. Both oils contain α -copaene in low concentration (0.9% for angelica seed oil [Flath et al. 1994a] and 0.4% for ginger root oil [T. W. Phillips, personal communication]) with the positive enantiomer predominating (98.6% (+) enantiomer for angelica seed oil [Flath et al. 1994a] and 81% (+) enantiomer for ginger root oil [Takeoka et al. 1990]). Both oils contain additional sesquiterpenes whose effect on *C. capitata*, either independently or in combination with α -copaene, remain largely unknown (but see Flath 1994a, 1994b).

Male Mating Success. For both α -copaene and angelica seed oil, the effect of exposure on male mating success was examined in a single experiment in which treated males were exposed to the chemical when sexually mature (9–11 d old) and tested 2 d later. I applied 20 μ l of the hexane elution of pure α -copaene or the angelica seed oil to a small disc of filter paper using a microcapillary pipette. The disc was placed on the bottom of a transparent, plastic drinking cup (400 ml volume), 25 males were immediately placed in the cup using an aspirator, and the cup was covered with nylon screening. Exposure started at 0700 hours and continued until 1300 hours. The behavior of males was not monitored systematically during exposure periods, but in frequent checks males were never observed touching the paper discs containing either α -copaene or angelica seed oil, a finding consistent with Nishida et al. (2000). Rather, the chemical acted as an arrestant, and males were generally quiescent. After exposure, treated males were removed from the "exposure cups," placed in holding buckets, and moved to an adjacent room. In all instances, the exposure procedure was conducted in a room isolated from any flies to prevent the inadvertent exposure of control males.

For ginger root oil, I ran a total of six experiments. The first was identical to those described above, i.e., males were exposed to the oil (20 μ l in this and all following experiments) when 10–12 d old following the standard procedure (described above) and tested 2 d later. Second, males were exposed to the oil when 9–12 d old for a reduced period (1 h) and tested 2 d later. Third, treated males were exposed to the oil when 10–12 d old for 1 h and held 5 d before testing. Fourth, treated males were exposed to the oil when sexually immature (1 d old) for 6 h and tested 8–10 d

later. Fifth, to confirm that the observed changes in mating competitiveness were effected by aroma alone and did not require direct contact with the attractants, I ran an experiment in which the oil-containing discs were not placed directly in the plastic cups but were placed in small containers (covered with nylon mesh screening) that were introduced into the plastic cups. Thus, males were exposed to the chemical's aroma but were unable to contact the source directly. In this experiment, males were exposed for 6 h when 9–11 d old and tested 2 d later. Finally, in the sixth experiment, I exposed pupae (2 d before eclosion) to the oil for 6 h and tested the males at 8–10 d after emergence. During exposure, 25 pupae were placed in cups near, but not in contact with, the oil-containing disc (pupae were not sexed; females were discarded upon emergence).

Mating tests were conducted at the Agricultural Experiment Station of the University of Hawaii, Waimanalo, Oahu. Groups of 100 treated (exposed to attractant) and 100 control (nonexposed) males and 100 females were released between 0730 and 0800 hours in field-cages (height: 2.5 m, diameter: 3.0 m) that contained a single rooted guava tree. For a given trial, I marked either treated or control males, alternating the marked group between successive trials. Males were marked 1 d before testing by cooling them for several minutes and placing a dot of enamel paint on the thorax. This procedure had no obvious adverse effects, and males resumed normal activities within minutes of handling. The cages were monitored continuously for 3 h, mating pairs were collected in vials, and the males identified. In all experiments, treated and control (i.e., nonexposed) males were of similar age when tested, and the females used were 9–13 d of age. Individuals of both sexes were used for only one trial.

Male Calling and Female Attraction. In an additional experiment, I examined whether ginger root oil affected male calling activity and long-range attraction of females. Data were collected in a large field tent (15 m long by 6 m wide by 2.5 m high) that included 17 rooted guava trees in two rows running the length of the tent. Groups of five males of a given type (control or treated) were placed in transparent, plastic cups (400 ml volume with both ends covered with wire screening) and suspended in four trees, with two having control males exclusively and two having treated males exclusively. Treated males were exposed to the oil when 9–12 d old following the standard procedure and tested 2 d later. Three cups, containing a total of 15 males of a given type, were placed on each of the trees at 0730–0800 hours ≈ 1.7 m above ground in shaded sites in the outer canopy. Cups on a given tree were placed in the same area of the canopy, so that distances between them did not exceed 0.3 m. The test trees were located at the corners of the tent.

Ten minutes after male placement, 100 females (9–14 d old) were released from the center of the tent. After an additional 10 min, the number of calling males and females at each cup were recorded at 10-min intervals for 120 min (i.e., $n = 13$ observations per tree

per replicate). Females that were perching on or within 15 cm of a cup were collected with an aspirator and placed in a holding bucket. This procedure appeared to have negligible impact on male calling activity or the behavior of other females. Tests were conducted on 11 d under sunny or partly sunny conditions with temperatures ranging from 22 to 26.5°C. The same release point and the same four trees were used in all replicates. For each tree, the type of male present (control or treated) was alternated between successive trials. Because some females invariably remained in the tent after a replicate (i.e., did not respond to the calling males), females were marked with enamel paint on the previous day (following the above procedure) to allow identification of those females released on a given test day. If sighted, females released on previous test days were collected but not included in the counts.

Statistical Analyses. The number of matings obtained by control and treated males was compared over all cages using a Mann-Whitney test (test statistic T). Because this test does not explicitly test for deviation from random mating (i.e., 50% of the matings by each male type), a binomial test (using the normal approximation with test statistic Z) was performed using data pooled over all cages. In the female attraction experiment, data from the three cups in the same tree were combined for each observation, and these values were averaged over all observations (13 per replicate) to obtain a single measure of male calling and female sightings for a given replicate. These summary values were used to compare treated and control males in a Mann-Whitney test.

Results

Male Mating Success. Males exposed to α -copaene obtained an average of 30.8 matings (range, 23–38) per replicate compared with only 15.1 (range, 10–19) for control males ($T = 100.0$; $n_1 = n_2 = 8$; $P < 0.001$). Over all replicates, males exposed to α -copaene accounted for 67% (247/368) of the matings ($Z = 6.9$; $P < 0.001$).

Males exposed to angelica seed oil achieved, on average, 31.6 matings (range, 24–38) per replicate compared with 10.0 matings (range, 5–17) for control males ($T = 77.0$; $n_1 = n_2 = 7$; $P < 0.001$). Over all replicates, males exposed to angelica seed oil accounted for 74% (221/300) of the matings ($Z = 9.3$; $P < 0.001$).

In the tests using ginger root oil, treated males obtained significantly more matings than control males in all experiments in which males were exposed as adults, i.e., experiments 1–5 (Table 1). Binomial tests for random mating revealed significantly biased mating frequencies favoring treated males in these experiments. Treated males accounted for the following proportion of total matings ($P < 0.001$ in all cases): experiment 1: 81% (380/472; $Z = 16.9$), experiment 2: 79% (276/351; $Z = 13.1$), experiment 3: 68% (284/420; $Z = 7.8$), experiment 4: 76% (400/525; $Z = 13.9$), and experiment 5: 78% (445/571; $Z = 16.1$). In the experiment involving pupal exposure to the oil, treated and

Table 1. Effect of ginger root oil on the mating success of male *C. capitata* under different conditions of exposure (see text for details)

Experiment	Conditions of exposure			Post-exposure interval, d	Matings per replicate		T
	Male age, d	Duration, h	Contact possible?		Treated	Control	
1	10–12	6	Yes	2	47.5 (38–60)	11.5 (2–27)	100.0*
2	9–12	1	Yes	2	30.7 (21–37)	8.3 (2–16)	126.5*
3	10–12	1	Yes	5	31.6 (19–44)	14.8 (5–20)	123.0*
4	1	6	Yes	8–10	44.4 (21–65)	13.9 (7–18)	126.0*
5	9–11	6	No	2	49.4 (38–61)	14.0 (2–23)	126.0*
6	Pupae	6	No	8–10	13.9 (9–20)	16.2 (9–43)	87.0NS

Numbers of matings are means; ranges are given in parentheses. Eight replicates were conducted for experiment 1; nine replicates were conducted for experiments 2–6.

* $P < 0.001$; NS, not significant.

control males obtained a similar number of matings (experiment 6, Table 1), and no deviation from random mating was detected with the binomial test ($125/271 = 46\%$ matings by treated males; $Z = 1.1$; $P > 0.05$).

Male Calling and Female Attraction. On average, 8.4 treated males (range, 6.5–10.8) were calling within an aggregation (tree) per observation compared with 6.5 control males (range, 2.8–9.1; $T = 613.5$; $n_1 = n_2 = 22$; $P < 0.01$; Fig. 1). Correspondingly, the average

number of females sighted per observation was significantly greater for aggregations of treated males ($\bar{x} = 1.25$; range, 0.2–2.1) than control males ($\bar{x} = 0.9$; range, 0.3–1.9; $T = 585.0$; $n_1 = n_2 = 22$; $P < 0.05$; Fig. 1). However, when compared on a per calling male basis, female arrivals were found to be identical between treated ($\bar{x} = 0.14$; range, 0.03–0.23) and control ($\bar{x} = 0.14$; 0.06–0.29) males ($T = 498.5$; $n_1 = n_2 = 22$; $P > 0.05$).

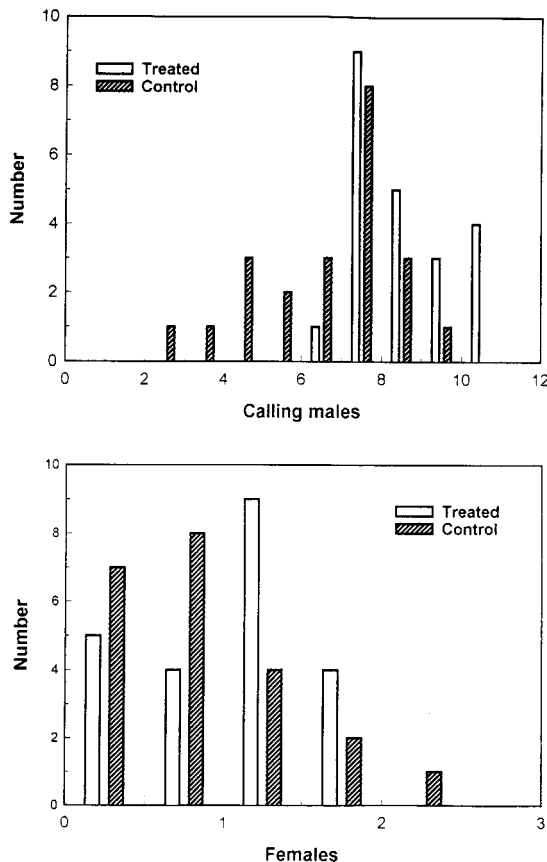


Fig. 1. Frequency distribution of calling males (top) and visiting females (bottom) for artificial leks of treated and control males ($n = 22$ leks for both male types). In both plots, values along the abscissa represent average numbers per observation ($n = 13$ observations per lek per replicate).

Discussion

Adult exposure to α -copaene, angelica seed oil, and ginger root oil significantly increased the mating success of male Mediterranean fruit flies. Mature males exposed to these substances for 6 h and tested 2 d later obtained 67–81% of the total matings. Similar results were observed for ginger root oil following shortened exposure interval, exposure to aroma only (when direct contact was prevented), and exposure to immature males. Also, in contrast to trimedlure (Shelly et al. 1996b), adult exposure to ginger root oil had long-lasting effects, and males tested 5 d after exposure still had a significant mating advantage. Exposure to pupae had no apparent effect on male mating frequency.

In the female attraction experiment, males exposed to ginger root oil spent more time pheromone-calling than nonexposed males. However, the finding that female abundance per calling male was similar between treated and control males suggests that pheromone quality (i.e., attractiveness per se) was unaffected by exposure to ginger root oil. This same result—enhanced signaling but unchanged pheromone attractiveness—was also reported for trimedlure-exposed, wild males (Shelly et al. 1996b). The structural differences between trimedlure or α -copaene and the major components of the male sex pheromone make it unlikely that either of these attractants is used in pheromone production (Millar 1995).

The increased mating success of treated males probably derived, in part at least, from their elevated calling levels. Although pheromone-calling was not monitored in the mating trials described here, previous studies (Whittier et al. 1994, Shelly 2000b) have documented a direct relationship between the calling activity of individual males and their mating fre-

quency. It is not known whether the attractants affected mating success in any other way. Data on the mating frequency of trimedlure-exposed, sterile males suggest that some other factor(s) are also important. In that study (Shelly 1999), nonexposed and exposed, sterile males displayed similar calling levels (i.e., trimedlure had no detectable effect on the calling level of mass-reared flies), which were significantly greater than those observed for wild males. Despite similar calling activity, however, nonexposed, sterile males were outcompeted by wild males, whereas exposed, sterile males outcompeted wild males. Thus, signaling activity alone apparently did not determine mating success, and trimedlure may have affected some other key factor(s), such as behavioral displays or olfactory signals produced during courtship.

Although the current experiments collectively revealed a pronounced mating skew favoring treated males, it is not known whether plant odors mediate male mating success in natural populations. α -Copaene apparently occurs in very low concentrations in plants. For example, Nishida et al. (2000) estimated that, by weight, α -copaene constituted 0.0001–0.0004% of leaves from various *Citrus* species, and McInnis and Warthen (1988) reported similar values for *Litchi chinensis* Sonn. and various *Ficus* species. Low leaf content has, in fact, prompted speculation that α -copaene acts primarily as a short-range attractant that induces male settlement in particular host species (McInnis and Warthen 1988, Nishida et al. 2000).

Although the treated males in the current study were most likely exposed to concentrations of α -copaene far above those found in many plants, there have been several reports of aggregations of *C. capitata* males on potential α -copaene-containing substrates. These observations include the following: (1) several hundred males clustered on leaf litter beneath a citrus tree (two different instances; Kaneshiro et al. 1995); (2) 5–10 males grouped tightly around milky exudate on a stem of *Ficus benjamina* L., a substance subsequently found to contain α -copaene (McInnis and Warthen 1988); (3) 20–30 males clustered on a dried orange lying on the ground (three different instances; T.E.S., unpublished data); and (4) in field-caged guava trees, groups of 20–30 males occasionally form at certain sites along branches (often, but not always, near breaks or gashes in the bark). These reports suggest that, in certain natural circumstances, α -copaene occurs in sufficient concentration or with a particular set of other sesquiterpenes to have long-range attractancy (i.e., at least over several meters) to *C. capitata* males. The occurrence of male-only groups away from typical lek sites (i.e., canopy of host trees) indicates that these males were not mate-searching but were attracted to these sites for some other purpose. Such groups, therefore, could result from male attraction to α -copaene (and possibly other sesquiterpenes), with subsequent enhancement of their mating success.

α -Copaene-containing oils could potentially be used to increase the effectiveness of the sterile insect technique via prerelease exposure of sterile males

(purifying α -copaene is a laborious procedure, thus rendering it impractical for large-scale use). Preliminary data (T.E.S., unpublished data) indicate that exposure to ginger root oil increases the mating success of mass-reared, sterile males relative to wild males. Although these data are promising, additional tests are required to examine possible effects of exposure on male longevity. For example, in studying the effects of adult nutrition on the performance of sterile males of *C. capitata*, Kaspi and Yuval (2000) reported that the addition of protein to the diet increased mating success but reduced survival. In addition, to assess the general utility of prerelease exposure, tests should be conducted with different strains of mass-reared males from different rearing facilities throughout the world.

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